

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | |
|---|-----------|--|
| (51) International Patent Classification ⁶ : C12Q 1/70 | A1 | (11) International Publication Number: WO 95/27083 (43) International Publication Date: 12 October 1995 (12.10.95) |
| <p>(21) International Application Number: PCT/US95/04061</p> <p>(22) International Filing Date: 31 March 1995 (31.03.95)</p> <p>(30) Priority Data: 08/221,098 31 March 1994 (31.03.94) US</p> <p>(71) Applicants: THE SCRIPPS RESEARCH INSTITUTE [US/US]; 10666 North Torrey Pines Road, La Jolla, CA 92037 (US). MAX-PLANCK-GESELLSCHAFT [DE/DE]; Generalverwaltung, Koniginstrasse 19, D-80539 Munchen (DE). VIRGINIA COMMONWEALTH UNIVERSITY [US/US]; MCV Station, Box 980568, Richmond, VA 23298-0568 (US).</p> <p>(72) Inventors: MILICH, David, R.; 11649 Vernet Court, El Cajon, CA 92020 (US). MARUYAMA, Toshiyuki; 2-21-12-503, Koraku, Bunkyo-ku, Tokyo 112 (JP). SCHODEL, Florian; 8800 Maywood Avenue, Silver Springs, MD 20910 (US). PETERSON, Darrell; 4345 Round Hill Drive, Chesterfield, VA 23832 (US).</p> <p>(74) Agents: HAILE, Lisa, A. et al.; Fish & Richardson P.C., Suite 1400, 4225 Executive Square, La Jolla, CA 92037 (US).</p> | | <p>(81) Designated States: AU, CA, FI, JP, NO, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published <i>With international search report.</i></p> |
| <p>(54) Title: METHOD FOR DIAGNOSING CHRONIC HEPATITIS B VIRUS INFECTION</p> <p>(57) Abstract</p> <p>Human antibodies that bind to woodchuck hepatitis virus core antigen are elevated in chronic hepatitis B patients in comparison to acute hepatitis B patients. Immunoassays for detection of the level of anti-WHV core antigen antibodies is used to distinguish chronic from acute hepatitis B patients.</p> | | |

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|----|--------------------------|----|---------------------------------------|----|--------------------------|
| AT | Austria | GB | United Kingdom | MR | Mauritania |
| AU | Australia | GE | Georgia | MW | Malawi |
| BB | Barbados | GN | Guinea | NE | Niger |
| BE | Belgium | GR | Greece | NL | Netherlands |
| BF | Burkina Faso | HU | Hungary | NO | Norway |
| BG | Bulgaria | IE | Ireland | NZ | New Zealand |
| BJ | Benin | IT | Italy | PL | Poland |
| BR | Brazil | JP | Japan | PT | Portugal |
| BY | Belarus | KE | Kenya | RO | Romania |
| CA | Canada | KG | Kyrgyzstan | RU | Russian Federation |
| CF | Central African Republic | KP | Democratic People's Republic of Korea | SD | Sudan |
| CG | Congo | KR | Republic of Korea | SE | Sweden |
| CH | Switzerland | KZ | Kazakhstan | SI | Slovenia |
| CI | Côte d'Ivoire | LI | Liechtenstein | SK | Slovakia |
| CM | Cameroon | LK | Sri Lanka | SN | Senegal |
| CN | China | LU | Luxembourg | TD | Chad |
| CS | Czechoslovakia | LV | Latvia | TG | Togo |
| CZ | Czech Republic | MC | Monaco | TJ | Tajikistan |
| DE | Germany | MD | Republic of Moldova | TT | Trinidad and Tobago |
| DK | Denmark | MG | Madagascar | UA | Ukraine |
| ES | Spain | ML | Mali | US | United States of America |
| FI | Finland | MN | Mongolia | UZ | Uzbekistan |
| FR | France | | | VN | Viet Nam |
| GA | Gabon | | | | |

METHOD FOR DIAGNOSING CHRONIC HEPATITIS B VIRUS INFECTION

This invention was made with Government support under Grant No. AI-20720 awarded by the National Institute of Health. The Government has certain rights in this invention.

5 BACKGROUND OF THE INVENTION

1. *Field of the Invention*

The present invention relates generally to the hepatitis B virus (HBV) and specifically to a method for diagnosing chronic hepatitis B virus infection.

2. *Description of Related Art*

10 Infection with hepatitis B virus (HBV) is a worldwide public health problem, with chronic carriers accounting for approximately 10 percent of the population of Asia and Africa. Major causes of HBV-associated mortality include chronic active hepatitis, liver cirrhosis and hepatocellular carcinoma. Both chronic carriers and newly infected individuals are at risk of succumbing to such complications. One important transmission route is the
15 infection of newborn infants at parturition by mothers who have active infections or are chronic carriers. Other routes for transmission include contaminated blood or blood products used to treat other health problems.

Infection with HBV often results in subclinical or acute self-limited liver disease or can result in chronic long-term infection. Chronic HBV infection elicits a spectrum of disease
20 entities ranging from the most severe form of chronic active hepatitis (CAH) to less severe chronic persistent hepatitis (CPH) to the asymptomatic carrier (ASC) state. An array of diagnostic assays have recently been developed to aid the clinician in differentiating hepatitis B virus infections from other forms of viral hepatitis (i.e., HAV, HEV, HCV).

-2-

However, the ability to distinguish between an acute hepatitis B (AH-B) infection and symptomatic chronic hepatitis B (CH-B) infection is still problematic. This is especially true since CAH and CPH patients often demonstrate a cyclic pattern of hepatitis characterized by acute exacerbations (A.E.) of liver injury alternating with normal liver function.

After infection with HBV, large quantities of the virus and associated particles are present in the serum. During the symptomatic phases of infection, both acute and chronic HBV patients have elevated liver enzyme levels, possess the hepatitis B surface antigen (HBsAg) in their serum, and produce antibodies to the nucleocapsid antigen (HBcAg). Antibodies specific for the HBsAg or the hepatitis B e antigen (HBeAg) are not detected. The appearance of antibody to HBsAg is usually not observed until approximately two months following disappearance of circulating HBsAg. The viral particles present in the serum are known to shed their surface coat exposing the nucleocapsid, known as the core antigen (HBcAg). Antibody production to HBcAg occurs early in the course of the acute phase of HBV infection and can persist for many years, and chronically infected patients produce high titers of anti-HBc antibodies.

Previous studies have indicated that the nucleocapsids of the HBV and the woodchuck hepatitis virus (WHV) share a crossreactive epitope (Werner, *et al.*, *J. Virol.* 1:314, 1979; Millman, *et al.*, *Infection and Immunity* 2:752, 1982; Stannard, *et al.*, *J. Gen. Virol.* 64:975, 1983; Ponzetto, *et al.*, *Virus Research* 2:301, 1985). Prior to the present invention, it was not known whether human anti-HBc antibodies recognized woodchuck hepatitis nucleocapsid antigen (WHcAg) or if sera from patients at different stages of HBV infection could be distinguished based on differential reactivity to WHcAg.

In contrast to most viral infections, acute and chronic HBV infected patients often produce both IgM and IgG anti-HBc antibodies, therefore, the mere presence of IgM anti-HBc is not diagnostic of an acute infection. However, higher levels of IgM anti-HBc are generally produced during the acute phase as compared to chronic infection, and this quantitative difference has become the only serologic means of differentiating an acute

HBV infection from an A.E. of a chronic infection. The usefulness of IgM anti-HBc assays in the differentiation of acute from chronic HBV infection has also been questioned. The distinction between acute and chronic HBV infection is important in terms of prognosis and possible treatment modalities.

- 5 Thus, there exists a need for a simple, inexpensive and reliable assay to distinguish patients with acute HBV infection from those with chronic HBV infection. The present invention provides such an assay.

SUMMARY OF THE INVENTION

The present invention is based on the unexpected discovery that subjects with acute and chronic hepatitis B virus infection can be easily distinguished based on the level of antibody to HBcAg^w.

- 5 In one embodiment the invention provides an assay for diagnosing chronic HBV infection in a subject by detecting IgG anti-woodchuck hepatitis core antigen antibody in a subject.

In another embodiment, the above-described assay is performed in conjunction with detection of IgM HBcAg antibodies and the ratio of IgM HBcAg:IgG HBcAg^w antibodies is determined.

- 10 In yet another embodiment, the invention provides a series of assays which include detection of serum levels of anti-HBe antibody, HBeAg/anti-HBe immune complex (IC) and HBsAg/anti-HBs ICs, all of which are significantly elevated in chronic versus acute hepatitis B patients.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is a comparison between acute hepatitis B (AH-B) and chronic hepatitis B (CH-B) patient serum levels of anti-HBe (panel A), HBeAg/anti-HBe ICs (panel B) and HBsAg/anti-HBs ICs (panel C).

- 5 FIGURE 2 is a comparison between acute hepatitis B (AH-B) and chronic hepatitis B (CH-B) patient serum levels of anti-HBc, IgM anti-HBc, IgG anti-HBc^w, and IgM anti-HBc/IgG anti-HBc^w ratio. (Panel A = total anti-HBc; Panel B = IgM anti-HBc; Panel C = IgG anti-HBc^w; and Panel D = ratio of IgM anti-HBc/IgG anti-HBc^w.)

- 10 FIGURE 3 is an endpoint titration of anti-HBc^w IgG in AH-B (Δ-Δ) and CH-B (○-○) patient sera (▲-▲ = normal human serum (NHS)).

FIGURE 4 is the kinetics of antibody production to the HBcAg in AH-B and CH-B patients. Sequential serum samples from AH-B (panel A) and CH-B (panel B) were analyzed for IgM anti-HBc (●-●), IgG anti-HBc (▲-▲), and IgG anti-HBc^w (Δ-Δ).

- 15 FIGURE 5 is the serologic profile of a CH-B patient. The top panel shows standard serologic parameters.

FIGURE 6 is antigen inhibition analysis of CH-B patient sera. Panel A is anti-HBc^w and panel B is anti-HBc assayed by direct ELA. (HBeAg, ▲-▲; HBcAg, ○-○; and WHcAg, Δ-Δ).

- 20 FIGURE 7 shows the sensitivity of anti-HBc^w reactivity to denaturation of woodchuck HcAg. (Mab 2221, Δ-Δ; Ra-WHC, ▲-▲-▲; Ma-WHC, ▲- - -▲; Ha-HBc^w, ○-○).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a sensitive new assay which detects antibody to woodchuck hepatitis core antigen (WHcAg) and distinguishes chronic from acute hepatitis B infection. The assay is based on the unexpected finding that patients with chronic hepatitis B infection have elevated serum levels of a species of anti-HBc antibody that crossreacts with woodchuck hepatitis core antigen (i.e., anti-HBc^w). In another embodiment, the invention provides a series of assays which include detection of serum levels of anti-HBe antibody, HBeAg/anti-HBe immune complexes (IC) and HBsAg/anti-HBs ICs, all of which are shown in the present invention to be significantly elevated in chronic versus acute hepatitis B patients.

The invention provides a method for diagnosing chronic hepatitis B virus (HBV) infection in a subject comprising contacting a sample from a subject suspected of having chronic HBV with an epitope that contains the amino acid sequence of a woodchuck hepatitis virus antigen wherein the epitope binds with antibody to woodchuck hepatitis B core antigen and detecting the presence of antibody that reacts with woodchuck hepatitis B core antigen in the sample. The subject can be any animal and preferably is a human.

As used in this invention, the term "epitope" means any antigenic determinant on an antigen to which the paratope of an antibody binds. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

The method of the invention for detection of anti-woodchuck hepatitis B core antigen antibodies (anti-HBc^w) in a sample is performed *in vitro*, for example, in immunoassays in which the antibodies can be identified in liquid phase or bound to an antigen or epitope that is bound to a solid phase carrier. In addition, an antibody for detection of anti-woodchuck HBc antibodies in these immunoassays can be detectably labeled in various

ways. Examples of types of immunoassays which can be utilized to detect anti-woodchuck HBc antibodies in a sample, include competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA) and the sandwich (immunometric) assay. Detection of the antibodies using the method of the invention can be done utilizing immunoassays which are run in either the forward, reverse, or simultaneous modes, including competition immunoassays and immunohistochemical assays on physiological samples. Those of skill in the art will know, or can readily discern, other immunoassay formats without undue experimentation.

10 The woodchuck HBc antigen (WHcAg), or epitopes thereof, can be bound to many different carriers and used to detect anti-woodchuck HBc antibodies in a sample. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amyloses, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding antigen or epitopes, or will be able to ascertain such, using routine experimentation.

There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, and bio-luminescent compounds. Those of ordinary skill in the art will know of other suitable labels for binding to the monoclonal antibodies of the invention, or will be able to ascertain such, using routine experimentation. Furthermore, the binding of these labels to the antibodies used in the method of the invention can be done using standard techniques common to those of ordinary skill in the art.

For purposes of the invention, antibodies that bind to woodchuck HBcAg may be detected using anti-human IgG antibodies. Any sample containing a detectable amount of

woodchuck HBcAg antibody can be used. A sample can be a liquid such as urine, saliva, cerebrospinal fluid, blood, serum and the like. Preferably, the sample is serum.

The invention also provides a series of assays which include detection of serum levels of anti-HBe antibody, HBeAg/anti-HBe immune complexes (IC) and HBsAg/anti-HBs ICs, all of which are significantly elevated in chronic versus acute hepatitis B patients. The detection of these antigens and antibodies is performed according to methods described above for the detection of anti-woodchuck hepatitis virus antibodies. For example, to detect, anti-HBe antibody, HBe antigen or peptide containing an epitope can be fixed to a solid support and a species specific antibody which binds to the anti-HBe antibody added after the antigen and antibody in the sample react. Similarly, the immune complexes can be detected in a sample by reaction with a second antibody that binds to the antigen in the complex and a third antibody which binds to the antibody in the complex. For example, a non-competitive monoclonal antibody specific for HBsAg or HBeAg is fixed to a solid support and a serum sample is added followed by the addition of a labeled monoclonal antibody to human IgG.

The P/N ratio, which represents the level of antibody and ICs in the sample (P) as compared to the level of antibody and ICs in control sera, is preferably greater than about 2 as a lower limit or cut-off, and most preferably greater than 3. Preferably, the detection is performed by determining the absorbance (OD_{492}) value of the sample and the control sera. Those of ordinary skill in the art will know of other techniques for determining the level of antibody or IC's in a sample.

The materials for use in the assay of the invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement one or more container means such as vials, tubes, and the like, each of the container means comprising one of the separate elements to be used in the method. For example, one of the container means may comprise a woodchuck HBcAg or

woodchuck HBcAg epitope, and another container may comprise an anti-human antibody which is, or can be, detectably labelled.

5 The types of assays which can be incorporated in kit form are many, and include, for example, competitive and non-competitive assays. Typical examples of assays which can utilize the antibodies of the invention are radioimmunoassays (RIA), enzyme immunoassays (EIA), enzyme-linked immunosorbent assays (ELISA), and immunometric, or sandwich immunoassays.

10 The term "immunometric assay" or "sandwich immunoassay", includes simultaneous sandwich, forward sandwich and reverse sandwich immunoassays. These terms are well understood by those skilled in the art. Those of skill will also appreciate that antibodies according to the present invention will be useful in other variations and forms of assays which are presently known or which may be developed in the future. These are intended to be included within the scope of the present invention.

15 In performing the assays, it may be desirable to include certain "blockers" in the incubation medium (usually added with the labeled soluble antibody). The "blockers" are added to assure that non-specific proteins, proteases, or antiheterophillic immunoglobulins to anti-HBc^w immunoglobulins, for example, present in the experimental sample do not cross-link or destroy the antibodies on the solid phase support, or the radiolabeled indicator antibody, to yield false positive or false negative results. The selection of "blockers" therefore may add substantially to the specificity of the assays described in the present invention.

25 It has been found that a number of nonrelevant (i.e., nonspecific antibodies of the same class or subclass (isotype) as those used in the assays (e.g., IgG1, IgG2a, IgM, etc.) can be used as "blockers". The concentration of the "blockers" (normally 1-100 $\mu\text{g}/\mu\text{l}$) is important, in order to maintain the proper sensitivity yet inhibit unwanted interference by mutually occurring cross reactive proteins in the specimen.

-10-

The assays of the invention include using antibodies immunoreactive with WHcAg, HBcAg^w, HBcAg, HBeAg/anti-HBe ICs and HBsAg/anti-ABsAg ICs or fragments thereof. Monoclonal antibodies, such as those used in the present Examples, can be made by immunizing an appropriate host, such as a mouse, with antigen containing fragments
5 of the whole protein by methods well known to those skilled in the art (Kohler, *et al.*, *Nature*, 256:495, 1975). Antibodies with reactivity to ICs described herein are available commercially or can be produced as described in Maruyama, *et al.*, (*J. Immunol Meth.*, 155:65, 1992). The term antibody, as used in this invention, is meant to include intact molecules as well as fragments thereof, such as Fab and F(ab')₂, which are capable of
10 binding an epitopic determinant on the different HBV or WHV antigens.

The following examples are intended to illustrate but not limit the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may alternatively be adopted without resort to undue experimentation.

EXAMPLE 1

15

MATERIALS AND METHODS

1. Recombinant Antigens and Synthetic Peptides

Recombinant HBcAg of the *ayw* subtype, recombinant HBeAg of the *ayw* subtype, and recombinant WHcAg were derived from *E. coli* expression vectors as previously described (Schödel, *et al.*, *Vaccines 90*, Cold Spring Harbor Laboratory, pp. 193-198,
20 1990; Schödel, *et al.*, *J. Biol. Chem.*, 268:1332-1337, 1993; Schödel, *et al.*, *Vaccine*, 6:624-628, 1993 (shows the nucleotide/amino acid sequence for WHcAg)). Synthetic peptides derived from the WHcAg sequence were synthesized by the Merrifield solid-phase method, and were subjected to HPLC on a C18 reverse phase column. All peptides used eluted as a single major peak (90%). Synthetic peptides were produced in the

peptide laboratory of the R.W. Johnson Pharmaceutical Research Institute by G.B. Thornton (La Jolla, CA).

2. Antibodies

5 Murine polyclonal anti-HBc, anti-WHc and rabbit polyclonal anti-WHc were produced by immunization with recombinant HBcAg or WHcAg. Polyclonal rabbit anti-HBc was purchased from DAKO (CA). Monoclonal (Mab) anti-HBc (3105, 3120), anti-HBe (904, 905) and anti-HBeAg peptide (2221) antibodies were provided by M. Mayumi (Jichi Medical College, Japan) (Takahashi, *et al.*, *J. Immunol.*, 130:2903-2907, 1983; Imai, *et al.*, *J. Immunol.*, 128:69-72, 1982; Takahashi, *et al.*, *J. Immunol.*, 147:3156-3160, 1991).
10 These antibodies can be purchased from the Institute of Immunology, Japan. Monoclonal anti-HBc (440, 442), and anti-HBe (420, 422, 426) antibodies were obtained from Green Cross (Osaka, Japan). Peroxidase conjugated Mab anti-human IgG was provided by Ortho Diagnostics (NJ). Peroxidase-conjugated polyclonal goat anti-mouse or rabbit Ig was purchased from Boehringer Mannheim (IN).

15 3. Direct Solid-Phase Enzyme Immunoassays

Solid-phase enzyme immunoassays (ELA) were used to measure serum anti-HBc^w, IgG anti-HBc, IgM anti-HBc or anti-HBe levels. Recombinant WHcAg, HBcAg or HBeAg were coated onto microtiter plates (50 ng/well) overnight at 4°C, the plates were preincubated for 1 hour at 37°C with 50μl of PBS containing 1% BSA, 0.005% Tween
20 20, and 5% heat inactivated goat serum (blocking buffer). Human sera were diluted 1/500 in blocking buffer and added to the plates and incubated for 2 hours. The plates were incubated with 50μl of peroxidase conjugated anti-human IgG (1:3000) or anti-human IgM (1:1000) for 2 hours. The plates were developed by a final incubation for 10 min. with 50μl of orthophenylene diamine (OPD). The absorbance was read on an automatic
25 microtiter plate reader. The data are expressed as a P/N ratio, which represents the

-12-

absorbance (OD_{492}) value of the sample (P) as compared to the mean absorbance value of at least 15 control sera.

Quantitation of serum IgM anti-HBc was also performed by using the Abbott Corzyme-M kit (Abbott Labs, Chicago, IL) according to the manufacturer's procedure. A cut-off value of 0.25 times the positive control mean (PC) plus the negative control mean (NC) was calculated ($0.25 \times PC + NC$), and the sample/cut-off ratio was defined as the cut-off index (C.I.) according to the manufacturer. At the same time, quantitation of serum IgM anti-HBc was performed using the direct EIA. There was a high correlation in IgM anti-HBc values in sera tested in the Corzyme-M kit versus the direct EIA ($p < 0.001$, $r = 0.62$, $n = 86$).

Detection of total serum anti-HBc was also performed by using the Abbott Corzyme kit (Abbott Labs, Chicago, IL) according to the manufacturer's procedure. In this assay, anti-HBc was expressed as a percent inhibition, and inhibition of more than 50% is considered positive for anti-HBc.

4. Quantitative HBsAg or HBeAg-Specific Immune Complexes Assays

To detect HBsAg or HBeAg-specific immune complexes in human serum, a solid phase EIA has been developed (Maruyama, *et al.*, *J. Immunol. Meth.*, **155**:65-75, 1992). Briefly, plates were coated with monoclonal antibody ($0.5 \mu\text{g/ml}$) specific for either HBsAg (1B1E7F7) (Ortho Diagnostic, N.J.) or specific for HBe Ag-specific peptide (2221) as the capture reagents. These solid phase Mabs were chosen because they were previously demonstrated to bind immune-complexed as well as free antigen. Test sera diluted 1/25 in blocking buffer were added to the wells coated with solid-phase Mabs and the plates were washed after a 2 hr. incubation. The serum anti-HBs or anti-HBe antibody component of the IC was then detected with peroxidase-labeled monoclonal antibody to human IgG. The data are expressed as a P/N ratio, which represents the absorbance (OD_{492}) value of the sample (P) as compared to the mean absorbance value of at least 15 control sera.

5. Patients

Twenty-six patients with acute hepatitis type B (AH-B), fifty-three patients with chronic hepatitis-B (CH-B) including twelve patients with chronic persistent hepatitis (CPH) and forty-one patients with chronic active hepatitis (CAH) were studied. The diagnosis of AH-B was based on the findings of elevated values of serum alanine aminotransferase (ALT) (at least 10 times the upper limit of normal), associated with the detection of HBsAg and IgM anti-HBc antibodies in the serum together with the recent onset of jaundice and other typical symptoms of acute hepatitis. All AH-B patients were known to be HBsAg negative prior to liver injury. Furthermore, all AH-B patients recovered completely from illness, with normalization of ALT and clearance of HBsAg from the serum. All CH-B patients in this study who were persistently positive for HBsAg and HBeAg in their serum for more than 1 year, were diagnosed as CPH or CAH by histology. All AH-B patients were followed at the First Department of Internal Medicine, Tokyo University (Tokyo, Japan). All sera were tested for HBsAg/anti-HBs, HBeAg/anti-HBe, anti-HBc, IgM anti-HBc, IgM anti-hepatitis A virus, anti-HDV and anti-HCV by using commercial enzyme immunoassays (EIAs; Abbott Laboratories) in addition to the experimental assays for IgM anti-HBc, IgG anti-HBc, anti-HBe, anti-HBc^w, and HBsAg or HBeAg-specific immune complexes measured by direct solid-phase EIAs. All AH-B and CH-B patients were negative for IgM-anti-HAV, anti-HDV and anti-HCV. As controls for the novel anti-HBc^w assay, sera were collected from an additional 26 healthy controls, 7 acute hepatitis A, 8 acute hepatitis C, 30 chronic hepatitis C, and 10 HBsAg-positive and HBeAg-negative chronic hepatitis B patients.

HBV-DNA was determined using a dot blot procedure (Lieberman, *et al.*, *Hepatology*, 3:285-291, 1983). Briefly, 10 μ l of serum was denatured and applied to a nitrocellulose membrane under vacuum. Hybridization was performed using ³²P labeled cloned HBV-DNA (specific activities; 2-4 x 10⁸ cpm/ μ g). The membranes were autoradiographed for 96 hours on X-ray films. Serially diluted, known quantities of cloned HBV-DNA served as positive controls, and 0.5 pg of HBV-DNA was detectable in the assay.

Comparison between mean values in all assays was determined by Student's t-test. A p value (0.05) was considered significant.

EXAMPLE 2

COMPARISON OF THE PREVALENCE OF ANTI-HBe, HBeAg/ANTI-HBe ICs, AND HBsAg/ANTI-HBs ICs IN ACUTE AND CHRONIC HBV INFECTION

5

10

15

Serum samples from 26 acute hepatitis type B (AH-B) patients and 53 chronic hepatitis type B (CH-B) patients collected during period of peak alanine aminotransferase (ALT) elevations (mean ALT in AH-B patients 1790 ± 1351 U/l; in CH-B patients 385 ± 229 U/l) were analyzed for the presence of anti-HBe, HBeAg/anti-HBe immune complexes (ICs) and HBsAg/anti-HBs ICs by the experimental immunoassays described. Note that all AH-B and CH-B patient sera were negative for anti-HBe antibody when tested in a commercial assay (Abbott Laboratories). In contrast, when sera were analyzed by the direct EIA method all CH-B patients demonstrated significant anti-HBe antibody production (FIGURE 1,A).

20

FIGURE 1 shows a comparison between AH-B and CH-B patient serum levels of anti-HBe, HBeAg/anti-HBe ICs, and HBsAg/anti-HBs ICs. Serum samples from 26 AH-B and 53 CH-B patients were analyzed for (A) anti-HBe at a 1/500 dilution; (B) HBeAg/anti-HBe ICs at a 1/25 dilution; and (C) HBsAg/anti-HBs ICs at a 1/25 dilution in direct EIAs as described. Single serum samples were collected from each patient at the peak of ALT elevation representing the period of most severe liver injury. Results are expressed as a P/N (Positive/Negative) ratio and the mean \pm s.d. for each patient group is also shown. $N=0.016 \pm 0.0002$ (anti-HBe assay); $N=0.081 \pm 0.028$ (HBeAg/anti-HBe ICs assay); and $N=0.093 \pm 0.034$ (HBsAg/anti-HBs ICs assay).

Although most AH-B patient sera were positive for anti-HBe, the sera of AH-B patients contained significantly less anti-HBe antibody as compared to CH-B patients. However, 13% of CH-B and 46% of AH-B patients exhibited overlapping levels of anti-HBe between 7.9 and 24.1 P/N values. This degree of overlap compromises the usefulness of this assay in terms of discriminating between AH-B and CH-B infection. Because the sera of AH-B and CH-B patients taken during periods of liver injury were also positive for the HBeAg, sera were analyzed for the presence of HBeAg/anti-HBe ICs (FIGURE 1,B). The sera of CH-B patients contained significantly greater levels of HBeAg/anti-HBe ICs as compared to AH-B patient sera and the degree of overlap between the two patient groups was quite minimal (7% of CH-B and 19% of AH-B patients exhibited overlapping P/N values between 2.2 and 3.5). Similarly, CH-B patient sera contained significantly higher levels of HBsAg/anti-HBs ICs as compared to AH-B patient sera (FIGURE 1,C). However, there was a significant overlap in the HBsAg/anti-HBs IC assay between P/N values of 1.1 and 5.4 amongst CH-B patients (18%) and AH-B patients (92%).

Cumulatively, the experimental assays for the measurement of anti-HBe, HBeAg/anti-HBe ICs, and HBsAg/anti-HBs ICs illustrate very significant differences in the mean levels of these parameters between AH-B and CH-B patient sera, however, complete discrimination based on any single assay would be difficult due to overlapping values at the margins (FIGURES 1A-C). However, all AH-B and CH-B patient sera were analyzed at the same single dilution (i.e., 1/500 for anti-HBe analysis; and 1/25 for IC analysis). It is possible that modifications in assay design may yield results more useful in discriminating between acute and chronic HBV patient groups.

EXAMPLE 3**COMPARATIVE LEVELS OF IgG ANTI-HBc, IgM ANTI-HBc AND
ANTI-HBc^w IN ACUTE AND CHRONIC HBV INFECTION**

A commercial anti-HBc assay (Corzyme, Abbott Labs) modified by using 1/500 dilutions
5 of sera was used to compare AH-B and CH-B patient sera for total anti-HBc. FIGURE
2 shows a comparison between AH-B and CH-B patient serum levels of anti-HBc, IgM
anti-HBc, IgG anti-HBc^w, and IgM anti-HBc/IgG anti-HBc^w ratio. Serum samples from
26 AH-B and 53 CH-B patients were analyzed for (A) total anti-HBc at a dilution of
1/500 in the Corzyme assay (Abbott Laboratories); (B) IgM anti-HBc at a 1/1000 dilution
10 in the Corzyme-M assay (Abbott Laboratories); (C) IgG anti-HBc^w at a dilution of 1/500
in a direct EIA using WHcAg as the solid-phase ligand; and (D) the ratio of IgM anti-
HBc/IgG anti-HBc^w. Single serum samples were collected from each patient at the peak
of ALT elevation. Results are expressed as % inhibition in the anti-HBc assay, as a Cut-
Off Index (C.I.) and as a P/N ratio in the IgM anti-HBc assay, and as a P/N ratio in the
15 IgG anti-anti-HBc^w assay. The mean \pm s.d. for each patient group is also shown. $N=4.6$
 $\pm 9.5\%$ (IgG anti-HBc assay); $N=0.075 \pm 0.012$ and 1 C.I. was 0.298 (IgM anti-HBc
assay) and $N=0.014 \pm 0.002$ (IgG anti-HBc^w assay).

As previously shown, both AH-B and CH-B patients produce anti-HBc efficiently
although CH-B patient sera demonstrated higher levels ($101 \pm 3\%$ inhibition) as
20 compared to AH-B patient sera ($89 \pm 15\%$ inhibition) ($p < 0.001$). A direct EIA was also
used to measure IgG anti-HBc in AH-B and CH-B patient sera and the results were
similar to the commercial assay. Even using 1/500 serum dilutions in both anti-HBc
assays there was very significant overlap between AH-B and CH-B patients in terms of
IgG anti-HBc values (FIGURE 2, panel A). In contrast, use of the Corzyme-M kit
25 (Abbott Laboratories) to measure IgM anti-HBc antibody illustrates the preferential
production of IgM anti-HBc antibodies in AH-B patients as compared to CH-B patients
(FIGURE 2, panel B). The mean IgM anti-HBc level expressed as a Cutoff Index (C.I.)
was 9.2 ± 3.0 (or 37.0 ± 12.1 P/N ratio) in AH-B patient sera and 1.1 ± 0.9 C.I. (or $4.6 \pm$

4.0 P/N ratio) in CH-B patients ($p < 0.001$). Nonetheless, 20% of CH-B and 23% of AH-B patients exhibited IgM anti-HBc levels that overlapped between the 1.6 C.I. (6.6 P/N) and 6.3 C.I. (25.4 P/N) values.

5 Previous studies have indicated that the nucleocapsids of the HBV and the WHV share a crossreactive epitope(s) (Werner, *et al.*, *J. Virol.*, 1:314-322, 1979; Millman, *et al.*, *Infection and Immunity*, 2:752-757, 1982; Stannard, *et al.*, *J. Gen. Virol.*, 64:975-980, 1983; Ponzetto, *et al.*, *Virus Research*, 2:301-315, 1985). Therefore, it was of interest to determine if human anti-HBc antibodies recognized WHcAg, and if AH-B and CH-B patient sera could be distinguished based on differential reactivity to WHcAg. For this
10 purpose, a direct EIA was developed using recombinant WHcAg as solid-phase ligand and a monoclonal antibody (Mab) specific for human IgG as the probe.

FIGURE 3 illustrates the end-point titrations of pools of 5 AH-B patient sera, 5 CH-B patient sera and 5 normal human sera (NHS) assayed for IgG binding to solid-phase WHcAg. The data are expressed as mean \pm s.d. absorbance values (OD_{492}). The end-
15 point titer of the CH-B patient sera was 1/62,500 as compared to 1/100 in AH-B patient sera. This result suggested that at least a proportion of human anti-HBc antibodies can crossreact on WHcAg, and that chronically infected HBV patients may preferentially produce this crossreactive specificity designated as anti-HBc^w.

Based on the end-point titration analysis, a 1/500 serum dilution was chosen to screen the
20 26 AH-B and 53 CH-B patient sera for IgG binding to solid-phase WHcAg (FIGURE 2, panel C). The CH-B patient sera exhibited significantly higher levels of IgG anti-HBc^w (P/N 37.5 ± 10.5) as compared to AH-B patient sera (P/N 2.5 ± 1.4) ($p < 0.001$). Furthermore, there was no overlap between the IgG anti-HBc^w values of AH-B and CH-B patient sera (FIGURE 2, panel C). IgM anti-HBc^w antibody production was also
25 examined and no significant difference was observed between the low levels of IgM anti-HBc^w produced in AH-B patients (P/N 2.1 ± 1.6) and CH-B patients (P/N 2.4 ± 4.2).

-18-

The IgM anti-HBc assay and the IgG anti-HBc^w assays performed singly were the two most useful assays to discriminate between acute and symptomatic chronic HBV infection. However, performing both of these assays and expressing the results as a ratio of IgM anti-HBc/IgG anti-HBc^w provided a very powerful serologic method of discriminating between AH-B and symptomatic CH-B infections.

EXAMPLE 4

PRODUCTION OF ANTI-HBc^w IS UNIQUE TO HBV INFECTIONS

Before proposing the utilization of the anti-HBc^w assay to distinguish between acute and chronic HBV infections, it was necessary to establish that anti-HBc^w antibody production is specific to HBV infection and not a non-specific result of acute or chronic liver injury. In addition to the 53 HBeAg-positive CH-B patient sera previously described, 10 HBeAg-negative CH-B patient sera, 7 acute hepatitis A patient sera, 10 acute hepatitis C patient sera, 30 chronic hepatitis C patient sera, and 26 normal control sera were evaluated in the anti-HBc^w assay (Table 1). Only HBsAg-positive, CH-B patients positive or negative for the HBeAg produced anti-HBc^w antibody, and these patient groups produced anti-HBc^w at a frequency of 100%.

TABLE 1
PRODUCTION OF ANTI-HBc^w IgG
IS UNIQUE TO HBV INFECTION

| | Patients | No. | Mean \pm s.d. | Range | Anti-HB ^w |
|----|---------------------|-----|-------------------|---------------|----------------------|
| | | | | | Frequency (%) |
| 5 | HBsAg (+) | | | | |
| | HBeAg (+) CH-B | 53 | 0.556 \pm 0.156 | 0.141 - 0.957 | 100 |
| | HBeAg (-) CH-B | 10 | 0.494 \pm 0.266 | 0.131-1.003 | 100 |
| | HBsAg (-) | | | | |
| | Acute hepatitis-A | 7 | 0.012 \pm 0.002 | 0.009 - 0.015 | 0 |
| 10 | Acute hepatitis-C | 10 | 0.008 \pm 0.003 | 0.005 - 0.016 | 0 |
| | Chronic hepatitis-C | 30 | 0.008 \pm 0.002 | 0.004 - 0.017 | 0 |
| | Normal Controls | 26 | 0.007 \pm 0.003 | 0.006 - 0.014 | 0 |

15 Serum samples taken during periods of elevated ALT values from patients with the indicated diagnosis were diluted 1/500 and analyzed for IgG anti-HBc^w reactivity by direct EIA. Data are expressed as mean \pm s.d. absorbance (OD₄₉₂) values.

EXAMPLE 5
KINETICS OF IgM ANTI-HBc, IgG ANTI-HBc AND ANTI-HBc^w
ANTIBODY PRODUCTION IN ACUTE AND CHRONIC HBV INFECTION

20 To examine the kinetics of anti-HBc antibody production, temporal serum samples from 14 AH-B and 21 CH-B (4 CPH and 17 CAH) patients taken during a period of 4-5 months around the time of peak ALT elevations were analyzed for IgM anti-HBc, IgG anti-HBc and IgG anti-HBc^w. FIGURE 4 shows the kinetics of antibody production to the HBcAg in AH-B and CH-B patients. Sequential serum samples from 14 AH-B (A)

25 and 21 CH-B (B) patients were analyzed for IgM anti-HBc (•-•), IgG anti-HBc (▲-▲), and IgG anti-HBc^w (Δ-Δ) at the indicated time points relative to peak elevations in ALT values. All antibodies were measured by direct EIA. The results are expressed as mean

-20-

\pm s.d. P/N ratios. $N=0.015 \pm 0.005$ (IgM anti-HBc assay); $N=0.011 \pm 0.004$ (IgG anti-HBc assay); and $N=0.011 \pm 0.005$ (IgG anti-HBc^w assay).

In AH-B patients, IgM anti-HBc levels were initially high before the peak ALT elevation and declined thereafter (from a mean of 9.8 to 4.1 P/N), conversely, the production of IgG anti-HBc was initially low and increased during resolution of the hepatitis (from a mean of 9 to 27.5 P/N), and IgG anti-HBc^w production remained low to negative throughout the observation period (FIGURE 4,A). In CH-B patients, the level of IgM anti-HBc was relatively low 5-8 weeks prior to liver injury, but increased in parallel with ALT elevation and declined thereafter (from a mean of 3.0 to 6.2 P/N) (FIGURE 4B). The levels of IgG anti-HBc in CH-B patients remained quite high throughout the observation period (from a mean of 32.3 to 39.0 P/N), as did IgG anti-HBc^w production (from a mean of 43.0 to 50.3 P/N) (FIGURE 4,B). These data indicate that during the course of liver injury a single serum sample taken from an AH-B or CH-B patient may reflect fluctuating IgM and IgG anti-HBc antibody levels that may not clearly distinguish between an acute infection and an acute exacerbation of a chronic infection. In contrast, the anti-HBc^w levels remain consistently low in AH-B patients and consistently high in CH-B patients before, during and after periods of liver injury (FIGURE 4A, B).

The consistency and usefulness of the anti-HBc^w assay is demonstrated in the serological profile of a single CH-B patient shown in FIGURE 5. A CAH patient (H.M. a 22 year-old female) was monitored for a 6 year period during which two A.E. of liver injury (4/88 and 4/90) were recorded. The standard serologic parameters shown (top) were measured by commercial assays. IgM anti-HBc, IgG-anti-HBc, and IgG anti-HBc^w were measured by direct EIA as described. The data are expressed as P/N ratios for each time point (▲-▲ = IgM Anti-HBc; ●-● = ALT; ▲-▲ = IgG anti-HBc; ○-○ = IgM anti-HBc^w).

This CH-B patient experienced two A.E. of liver injury in April of 1988 and in April of 1990. If this patient appeared in the clinic for the first time in April of 1990 without a prior history of HBV infection, the only serological marker that would indicate an A.E.

of a chronic HBV infection rather than an acute HBV infection is the elevated anti-HBc^w value. Note that the IgM anti-HBc value is significantly elevated during the period of maximum liver injury when the patient is most likely to seek medical attention.

EXAMPLE 6

5 IMMONOLOGICAL CHARACTERIZATION OF THE HBc^w EPI TOPE

Although the production of IgG antibody reactive with WHcAg correlated with IgG anti-HBc antibody production in chronic HBV infection, it was necessary to confirm the antigenic relationship between the WHcAg and the HBcAg directly. For that purpose, a pool of sera obtained from 6 CH-B patients positive for anti-HBc^w was analyzed in an
10 antigen competition assay. FIGURE 6 shows an antigen inhibition analysis of CH-B patient sera. Sera from 6 CH-B patients positive for anti-HBc and anti-HBc^w antibodies were preincubated with the indicated concentrations of the soluble inhibitors HBcAg, WHcAg, or HBeAg overnight at 4°C. The residual anti-HBc^w (A) or anti-HBc (B) reactivity was then assayed by direct EIA using solid-phase WHcAg or HBcAg,
15 respectively, as solid-phase ligands. The data are expressed as percent binding compared to the absorbance value (OD₄₉₂) obtained without soluble inhibitor.

The CH-B patient sera was preincubated with soluble HBcAg, HBeAg or WHcAg prior to addition to either solid-phase WHcAg (FIGURE 6A) or HBcAg (FIGURE 6,B), and the percent of IgG binding to the solid-phase ligands as compared to unabsorbed CH-B
20 patient sera was determined. The binding of CH-B patient IgG to solid-phase WHcAg was quantitatively inhibited by WHcAg and HBcAg to the same degree but was not inhibited by HBeAg (FIGURE 6,A). This result indicates that the HBc^w epitope(s) recognized by CH-B patient IgG is present on both the HBcAg and the WHcAg but not on the HBeAg.

In the reciprocal assay, the binding of CH-B patient IgG to solid-phase HBcAg was quantitatively inhibited only by HBcAg and not by WHcAg or HBeAg (FIGURE 6,B). The inability of soluble WHcAg to inhibit the binding of CH-B patient IgG to HBcAg indicates that the HBc^w epitope(s) is crossreactive with only a minor proportion of
5 HBcAg epitopes, and that the HBc^w-specific antibody detected in CH-B patient sera represents only a minor component of the total IgG anti-HBc response. This is consistent with the observation that no HBcAg-specific Mab recognized the WHcAg (Table 2).

-23-

TABLE 2
THE HBc^w EPI TOPE IS NOT RECOGNIZED BY
THE KNOWN HBcAG AND HBeAG SPECIFIC
MONOCLONAL ANTIBODIES

| | Antibody | WHcAG | HBcAG | HBeAg |
|----|--------------------------------|-------|-------|-------|
| 5 | Mab Anti-HBc | | | |
| | 3105 | 0.01 | 1.29 | 0.16 |
| | 3120 | 0.04 | 1.99 | 0.08 |
| | 440 | 0.03 | 1.65 | 0.35 |
| | 442 | 0.01 | 0.58 | 0.11 |
| | Mab Anti-HBe | | | |
| | 904 | 0.01 | 0.08 | 0.92 |
| | 905 | 0.02 | 0.10 | 1.39 |
| | 420 | 0.01 | 0.12 | 1.93 |
| | 422 | 0.01 | 0.09 | 1.53 |
| | 426 | 0.01 | 0.03 | 1.43 |
| | Mab Anti-HBe peptide (2221) | 0.69 | 0.63 | 2.00 |
| | Poly Anti-HBc/e | 0.12 | 2.0 | 2.00 |
| 10 | Poly Anti-WHc | 1.90 | 0.16 | 0.11 |

15 Recombinant WHcAg, HBcAg and HBeAg were coated on the solid-phase (50ng/well), and the panel of indicated antibodies were assayed for binding in direct EIA. Mabs were assayed at 0.5 µg/ml and polyclonal sera was diluted 1/8000. The data are expressed as absorbance (OD₄₉₂) values.

Similarly, a panel of HBeAg-specific Mabs also failed to recognize the WHcAg. Only Mab 2221 specific for residues 129-140 of the HBc/HBe antigens bound WHcAg (Table 2). The 129-140 sequence is highly conserved between WHcAg and HBcAg.

5 Next, the requirement for native WHcAg structure for binding by human anti-HBc^w antibody was examined. Recombinant WHcAg was treated with a range of concentrations of SDS/2ME (0.006-6.4%), and human anti-HBc^w IgG, derived from a group of CH-B patient sera, and mouse anti-WHc and rabbit anti-WHc IgG, produced by immunization with rWHcAg, were analyzed for binding to solid-phase denatured WHcAg. FIGURE 7 shows sensitivity of anti-HBc^w reactivity to denaturation of
10 WHcAg. Recombinant WHcAg (1 mg/ml) was treated with the indicated concentrations of SDS/2ME for 2 hours at 37°C, and WHcAg (diluted 1000 X) was coated on the solid-phase (50 ng/well). Polyclonal mouse anti-WHc (Ma-WHc, ▲—▲) polyclonal rabbit anti-WHc (Ra-WHc) (▲—▲), monoclonal anti-HBe peptide (Mab 2221) (Δ-Δ), or human anti-HBc^w-positive antisera from 4 CH-B patients (Ha-HBc^w) (○-○) were added to the wells,
15 and bound antibodies were detected by peroxidase-labeled anti-murine, rabbit or human Ig. The data are expressed as percent binding compared to the absorbance value (OD₄₉₂) obtained without SDS/2ME treatment.

The Mab 2221, which binds the linear peptidic epitope 129-140, served as a positive control to insure that adequate denatured WHcAg adhered to the solid-phase well.
20 Binding of human anti-HBc^w to WHcAg was very sensitive to denaturation of WHcAg, and was reduced 80% by the treatment of WHcAg with 0.1% SDS/2ME. Similarly, the binding of polyclonal murine and rabbit antisera raised against native WHcAg were equally sensitive to the denaturation of WHcAg. The conformational-dependence of human anti-HBc^w was further demonstrated by the failure to detect binding of human
25 anti-HBc^w to a panel of WHcAg-derived overlapping peptides.

Lastly, to determine if the epitope(s) on WHcAg recognized by human anti-HBc^w IgG was species-specific, antibody competition assays were performed between human anti-

-25-

HBc^w antisera and murine and rabbit polyclonal antibody raised against rWHcAg (Table 3). The binding to WHcAg of a panel of 4 CH-B patient sera, positive for anti-HBc^w reactivity, was quantitatively inhibited by competitor rabbit and murine polyclonal anti-WHc antisera. Reciprocally, the binding to WHcAg of both rabbit and murine anti-WHc antisera were inhibited by a panel of 6 human anti-HBc^w-positive antisera used as competitors (Table 3). Therefore, the WHcAg crossreactive epitope(s) recognized by human anti-HBc^w-positive antisera is also recognized by mice and rabbits immunized with WHcAg.

TABLE 3**ANTI-HBc^w ANTIBODY PRODUCTION IS NOT SPECIES SPECIFIC**

| Primary Antibody | Competitor Antibody | Inhibition (%) | | |
|----------------------------------|----------------------------------|----------------|-------------|-------------|
| | | 1:100 | 1:500 | 1:2500 |
| H anti-HBc ^w (N=4) | R anti-WHc | 96.7 ± 0.9 | 96.0 ± 0.8 | 91.7 ± 2.7 |
| | M anti-WHc | 91.2 ± 1.8 | 87.2 ± 5.3 | 77.2 ± 8.5 |
| R anti-WHc | H anti-HBc ^w (N=6) | 40.7 ± 11.6 | 23.2 ± 17.0 | 17.2 ± 14.4 |
| M anti-WHc | H anti-HBc ^w (N=6) | 36.2 ± 10.1 | 23.2 ± 12.0 | 14.5 ± 8.8 |

The indicated competitor antibodies polyclonal rabbit (R) or murine (M) anti-WHc, or human (H) anti-HBc^w derived from CH-B patient sera were preincubated with solid-phase WHcAg (50 ng/well) for 2 hours at 37°C. Thereafter, the indicated primary antibodies were added to the wells and the percent inhibition by the competitor antibodies as compared to inhibition with preimmunization sera or normal human sera was determined.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that various changes and modifications can be made without department from the spirit or scope of the invention.

CLAIMS

1. A method of diagnosing chronic hepatitis B virus (HBV) infection in a subject comprising:
 - a. contacting a sample from a subject suspected of having HBV with an epitope that contains the amino acid sequence of a woodchuck hepatitis virus antigen wherein the epitope binds with antibody to woodchuck hepatitis B core antigen; and
 - b. detecting the presence of antibody to woodchuck hepatitis B core antigen in the sample.
2. The method of claim 1, wherein the sample is serum.
3. The method of claim 1, wherein the epitope is bound to a solid support.
4. The method of claim 1, wherein the detection includes the addition of second antibody that binds to the anti-woodchuck hepatitis B core antigen antibody.
5. The method of claim 4, wherein the second antibody is detectably labeled.
6. The method of claim 5, wherein the label is selected from the group consisting of enzymes, radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, phosphorescent compounds, and bioluminescent compounds.
7. The method of claim 1, wherein the anti-woodchuck hepatitis B core antigen antibody is isotype IgG.
8. The method of claim 7, further comprising detecting the level of IgM anti-hepatitis B core antigen in the subject.

- 5
9. The method of claim 8, wherein the ratio of IgM anti-hepatitis B core antigen antibody to IgG anti-woodchuck hepatitis B core antigen antibody is determined.
 10. The method of claim 1, wherein the chronic hepatitis is chronic acute hepatitis.
 11. The method of claim 1, wherein the chronic hepatitis is chronic persistent hepatitis.
 12. A method of diagnosing chronic hepatitis B virus (HBV) infection in a subject comprising:
 - a. contacting a sample from a subject suspected of having chronic HBV with a first reagent which binds with anti-HBe antibody, a second reagent which binds HBeAg/anti-HBe immune complexes (IC) and a third reagent which binds HBsAg/anti-HBs ICs; and
 - b. detecting the presence of anti-HBe antibody, HBeAg/anti-HBe immune complexes (IC) and HBsAg/anti-HBs ICs in the sample.
 13. The method of claim 12, wherein the sample is serum.
 14. The method of claim 12, wherein the reagent is an antigen or an antibody.
 15. The method of claim 12, wherein the reagent is bound to a solid support.
 16. The method of claim 12, wherein the reagent is detectably labeled.
 17. The method of claim 16, wherein the label is selected from the group consisting of enzymes, radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, phosphorescent compounds, and bioluminescent compounds.

18. The method of claims 12, wherein the detecting is measured as a positive to negative (P/N) ratio.
19. The method of claims 18, wherein the P/N ratio is greater than 3.
20. A reagent kit for diagnosing chronic hepatitis B infection comprising carrier means being compartmentalized to receive in close confinement therein one or more containers comprising a first container containing woodchuck hepatitis B core antigen epitope, and a second container containing an antibody that binds to a human antibody.
21. The kit of claim 20, wherein the antibody that binds to a human antibody is labeled.
22. The kit of claim 21, wherein the label is selected from the group consisting of enzymes, radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, phosphorescent compounds, and bioluminescent compounds.
23. A reagent kit for diagnosing chronic hepatitis B infection comprising carrier means being compartmentalized to receive in close confinement therein one or more containers comprising a first container containing HBc antigen, a second container containing an antibody that binds to HBeAg/anti-HBe IC and a third container containing an antibody that binds to HBsAg/anti-HBs IC.
24. The kit of claim 23, wherein the kit further contains a container with an antibody which binds to a human antibody.
25. The kit of claim 24, wherein the antibody that binds to a human antibody is labeled.

26. The kit of claim 25, wherein the label is selected from the group consisting of enzymes, radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, phosphorescent compounds, and bioluminescent compounds.

FIG. 1A

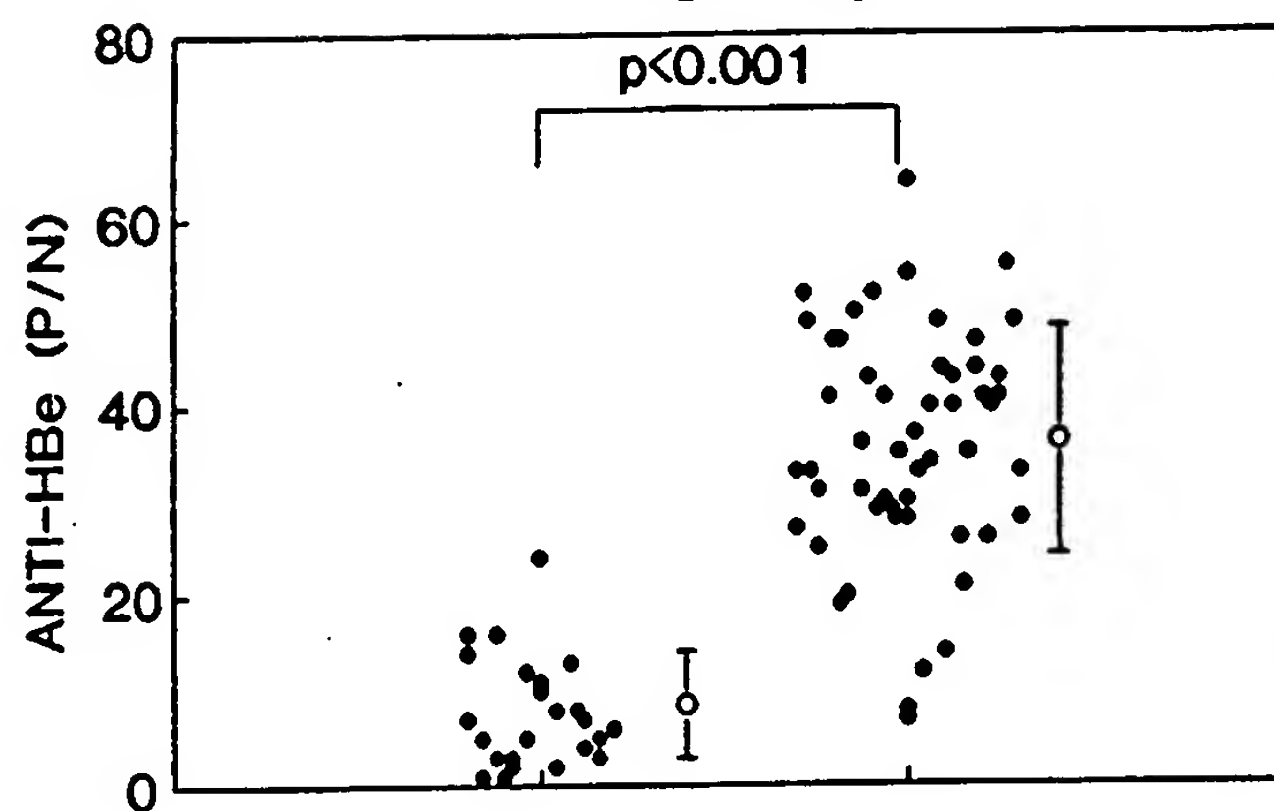


FIG. 1B

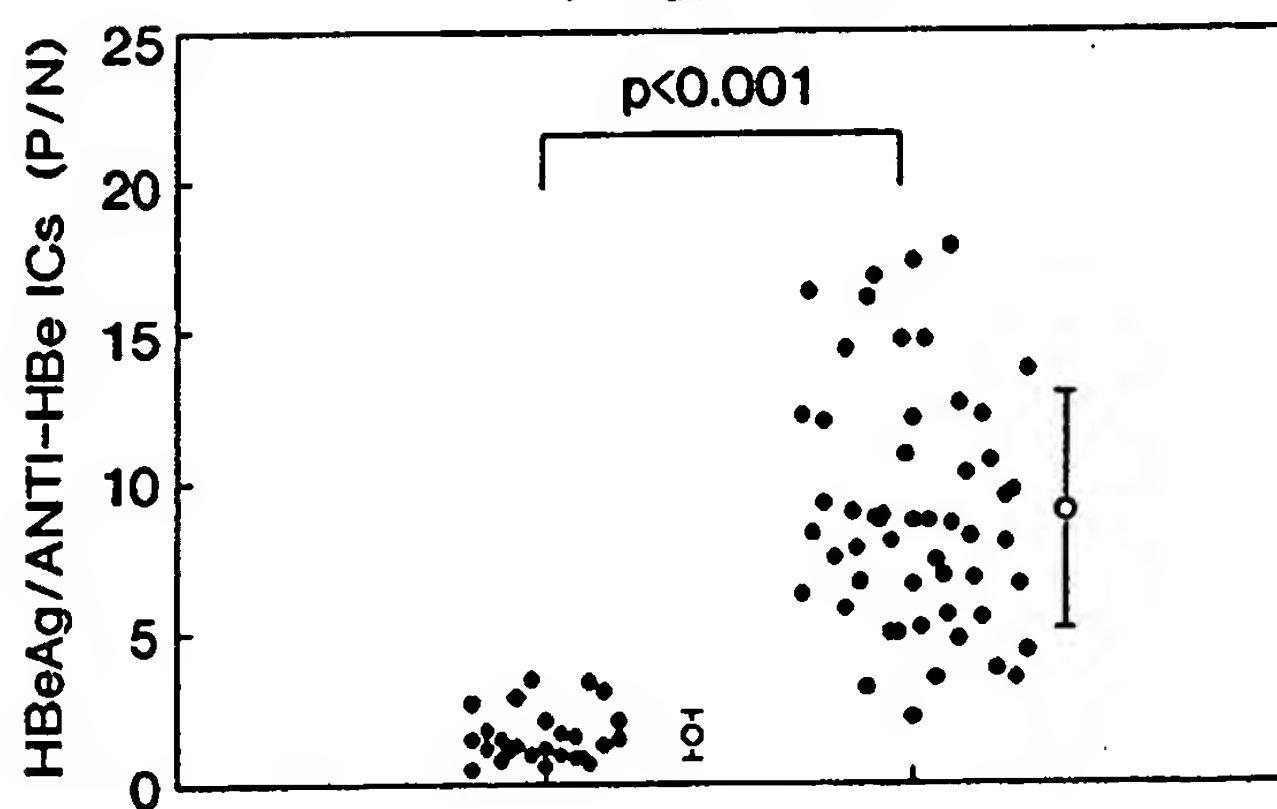
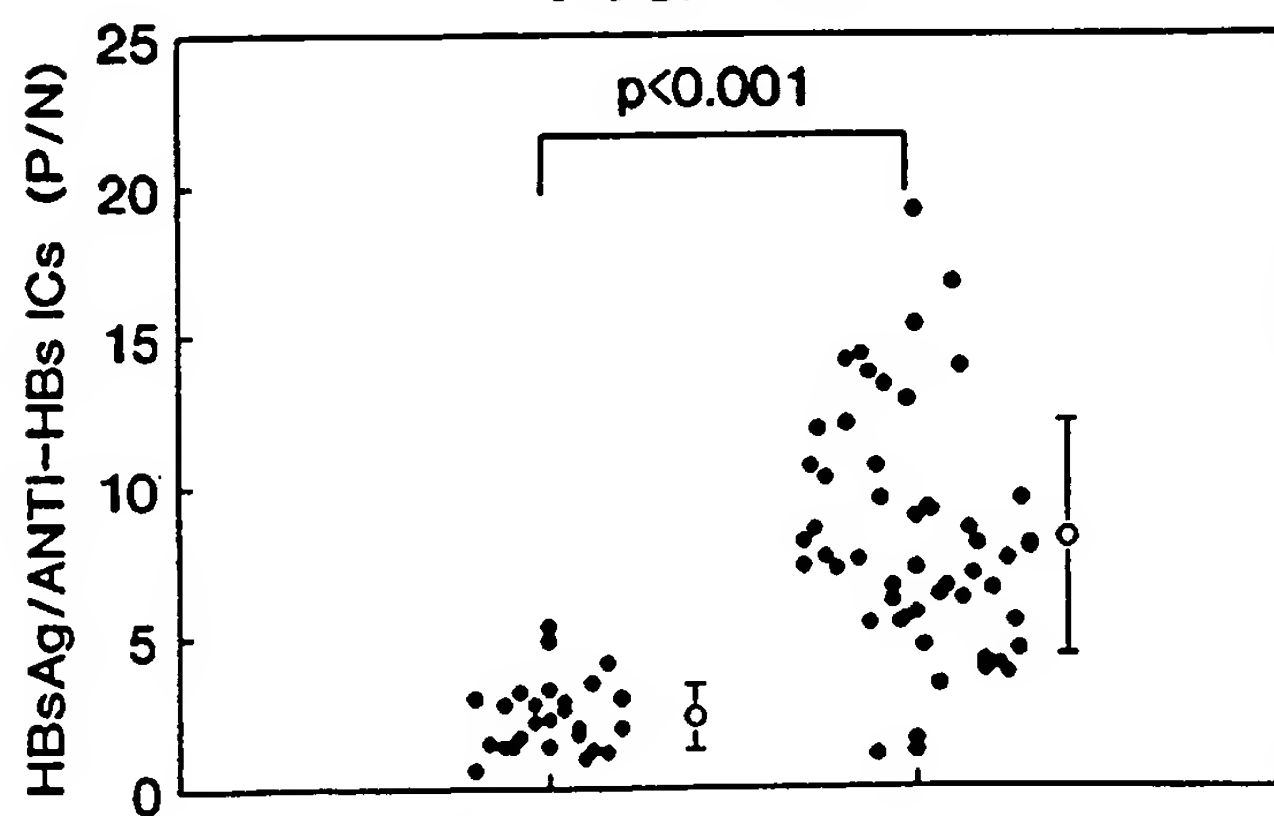


FIG. 1C



AH-B CH-B
SUBSTITUTE SHEET (RULE 26)

FIG.2A

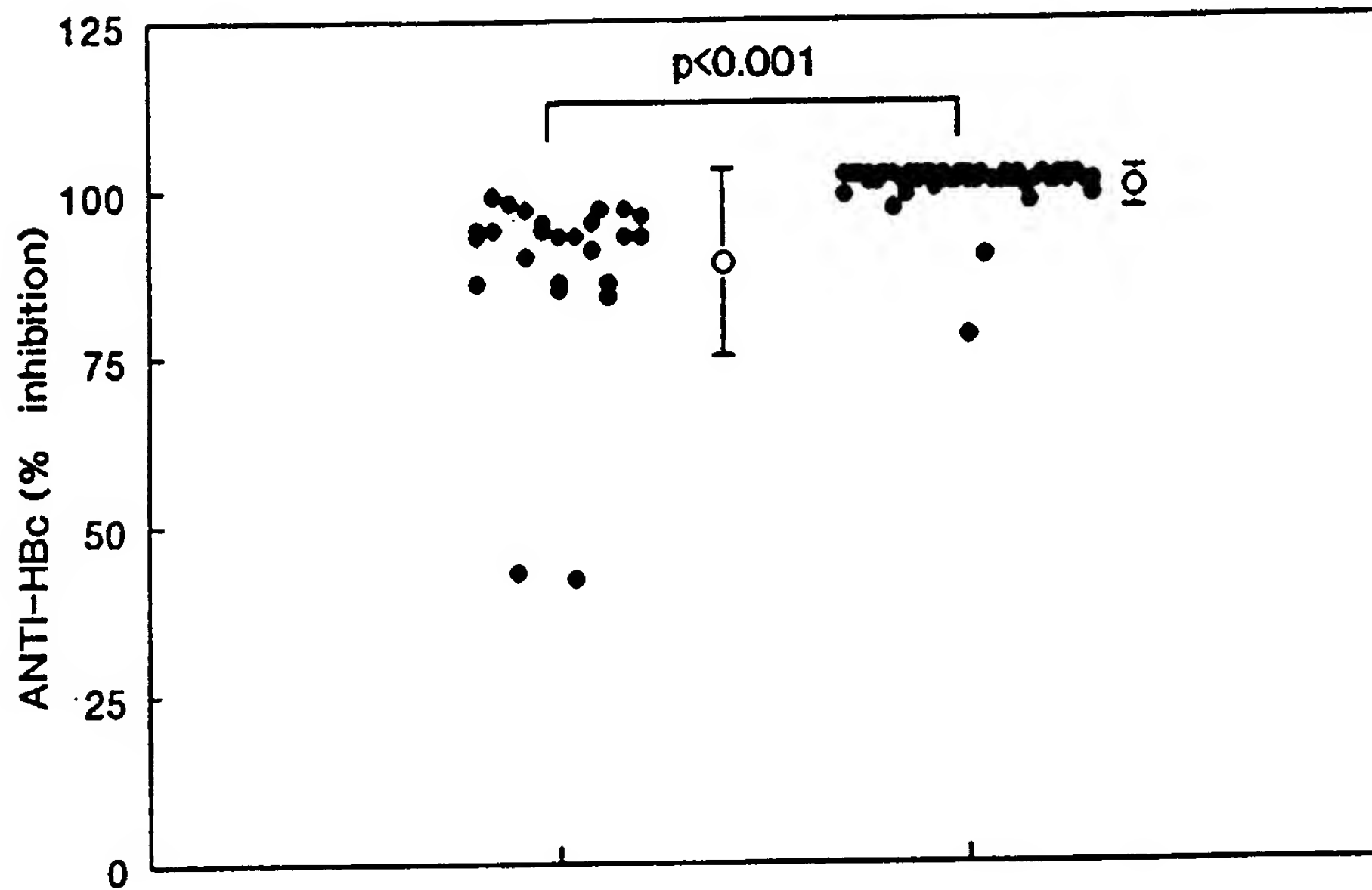


FIG. 2B

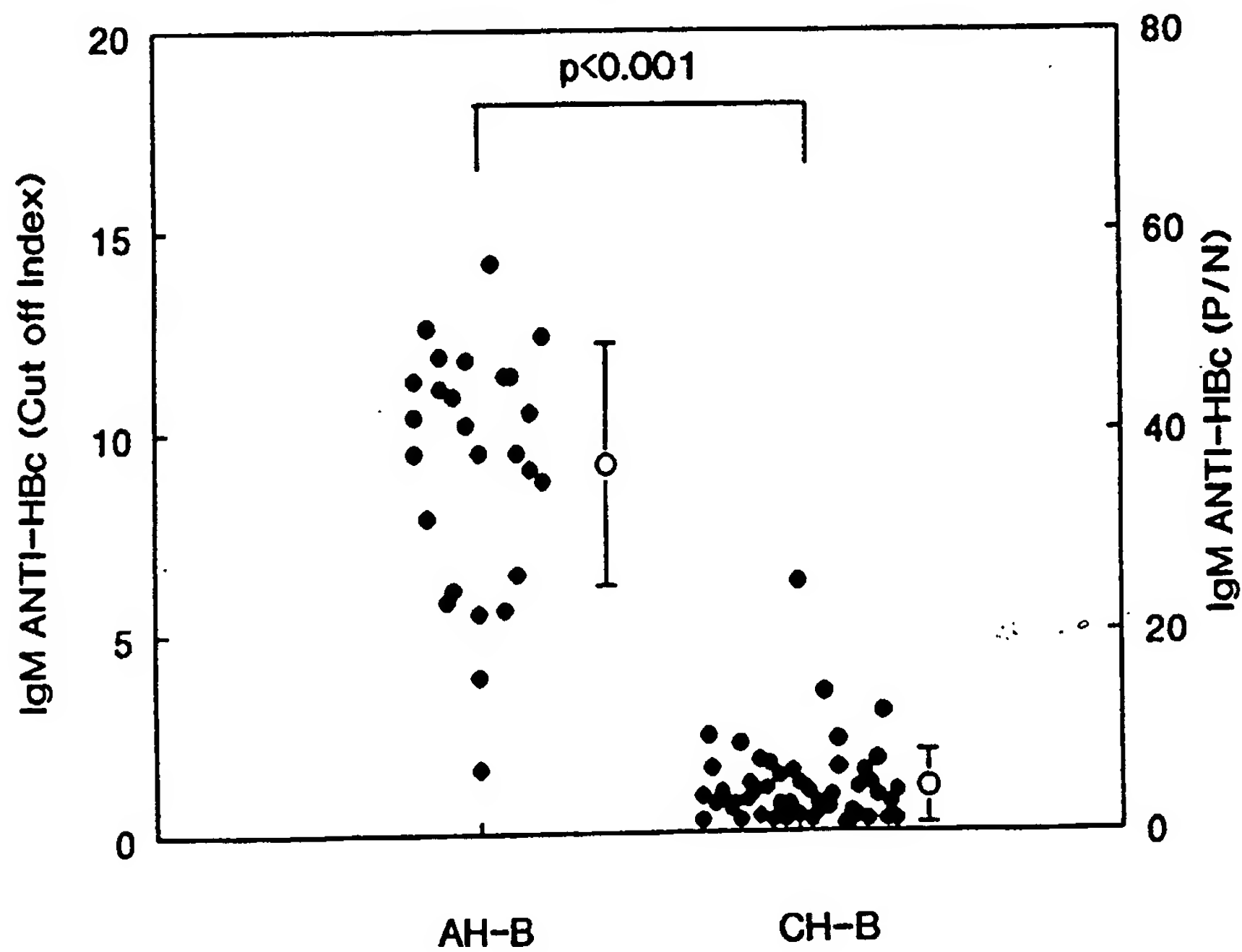


FIG. 2C

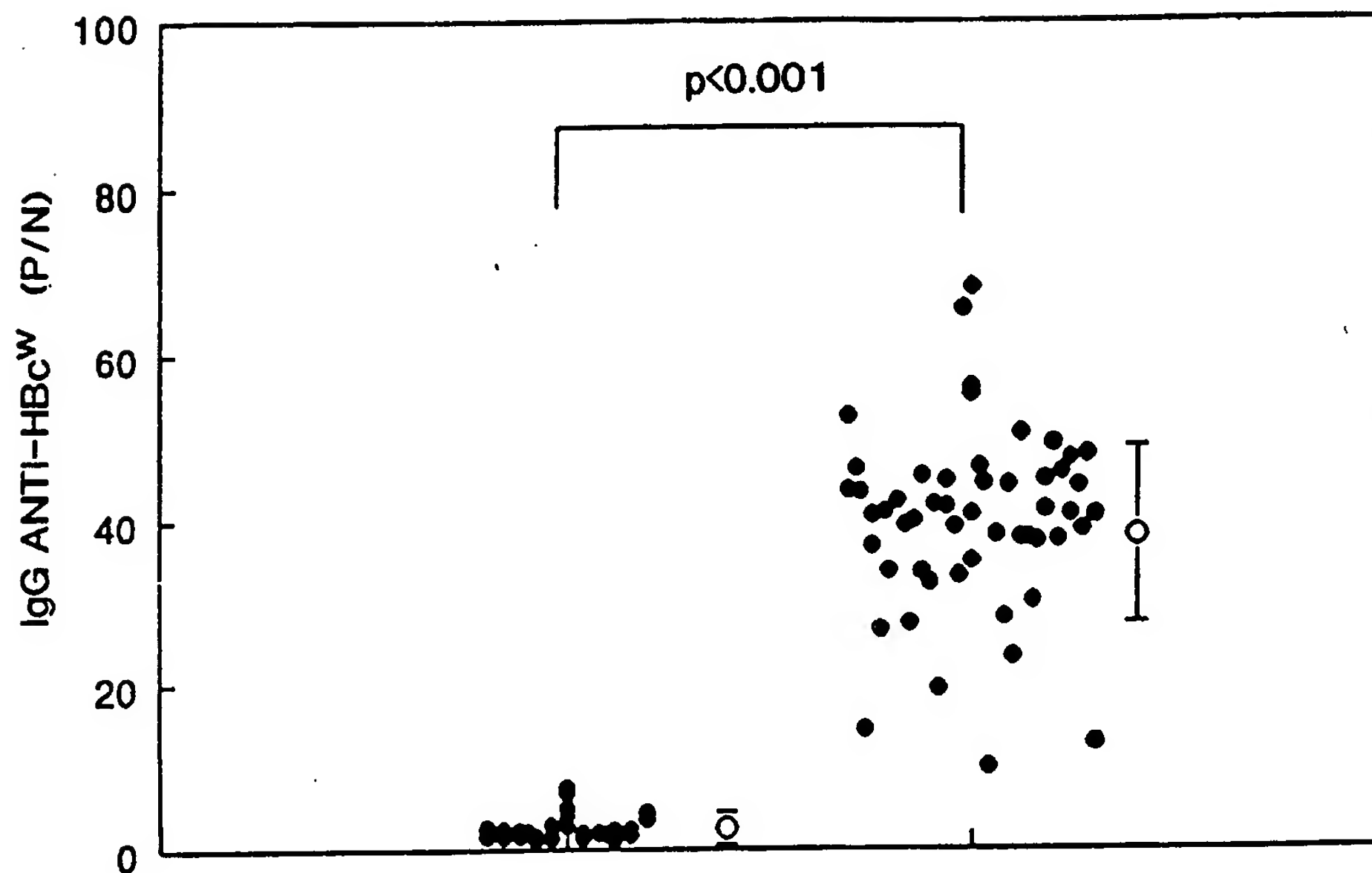
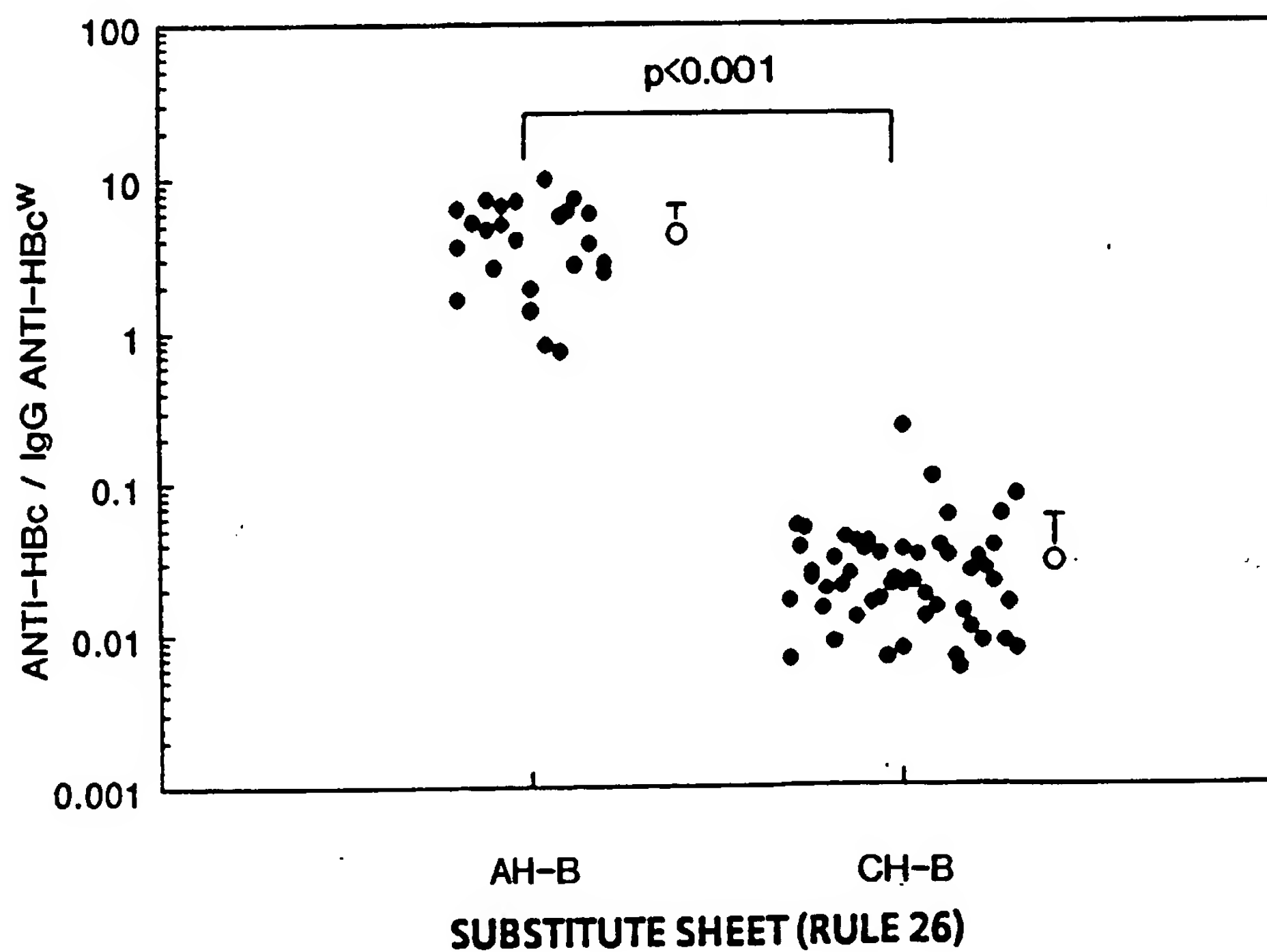


FIG. 2D



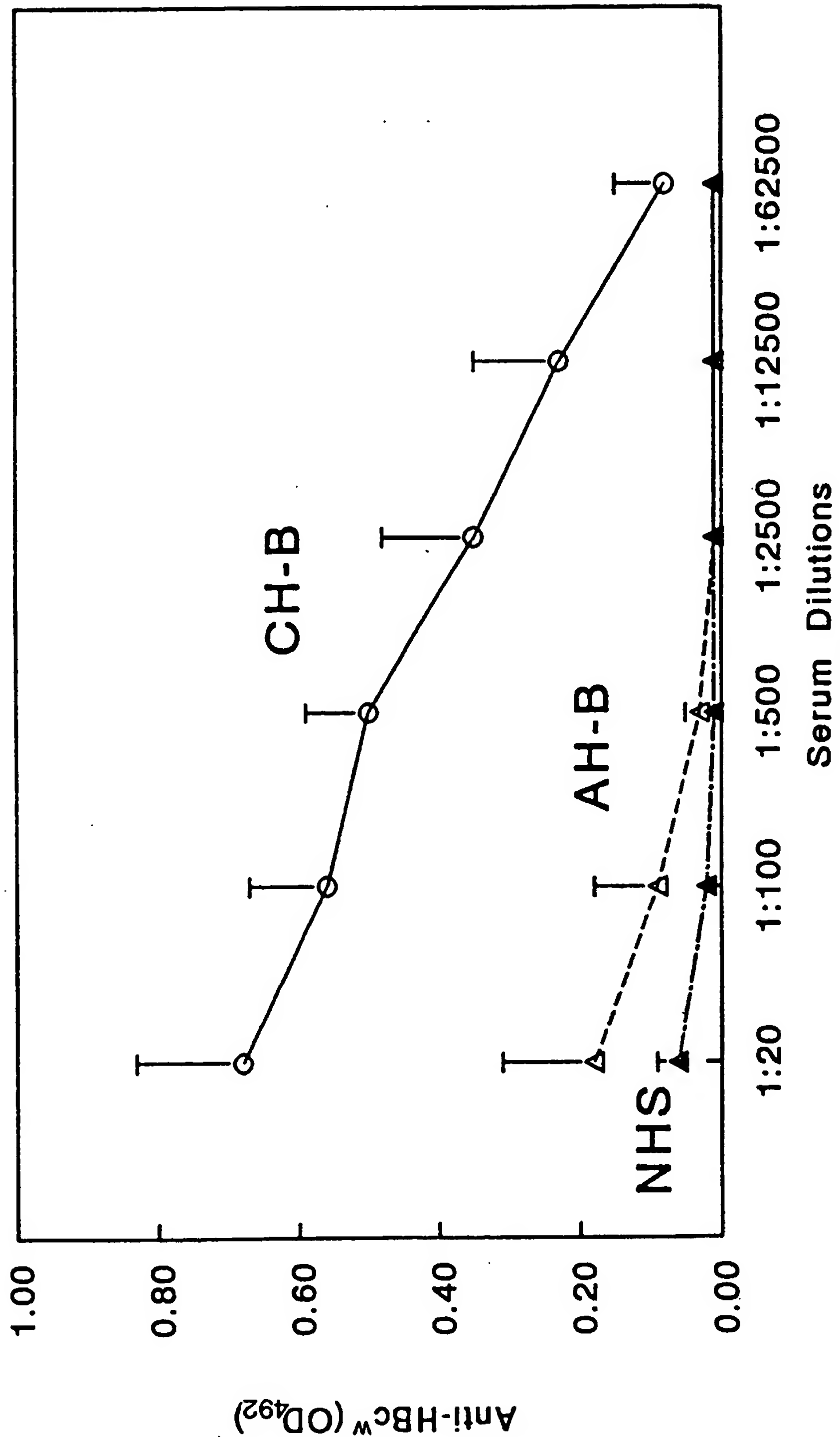


FIG. 3

FIG. 4A

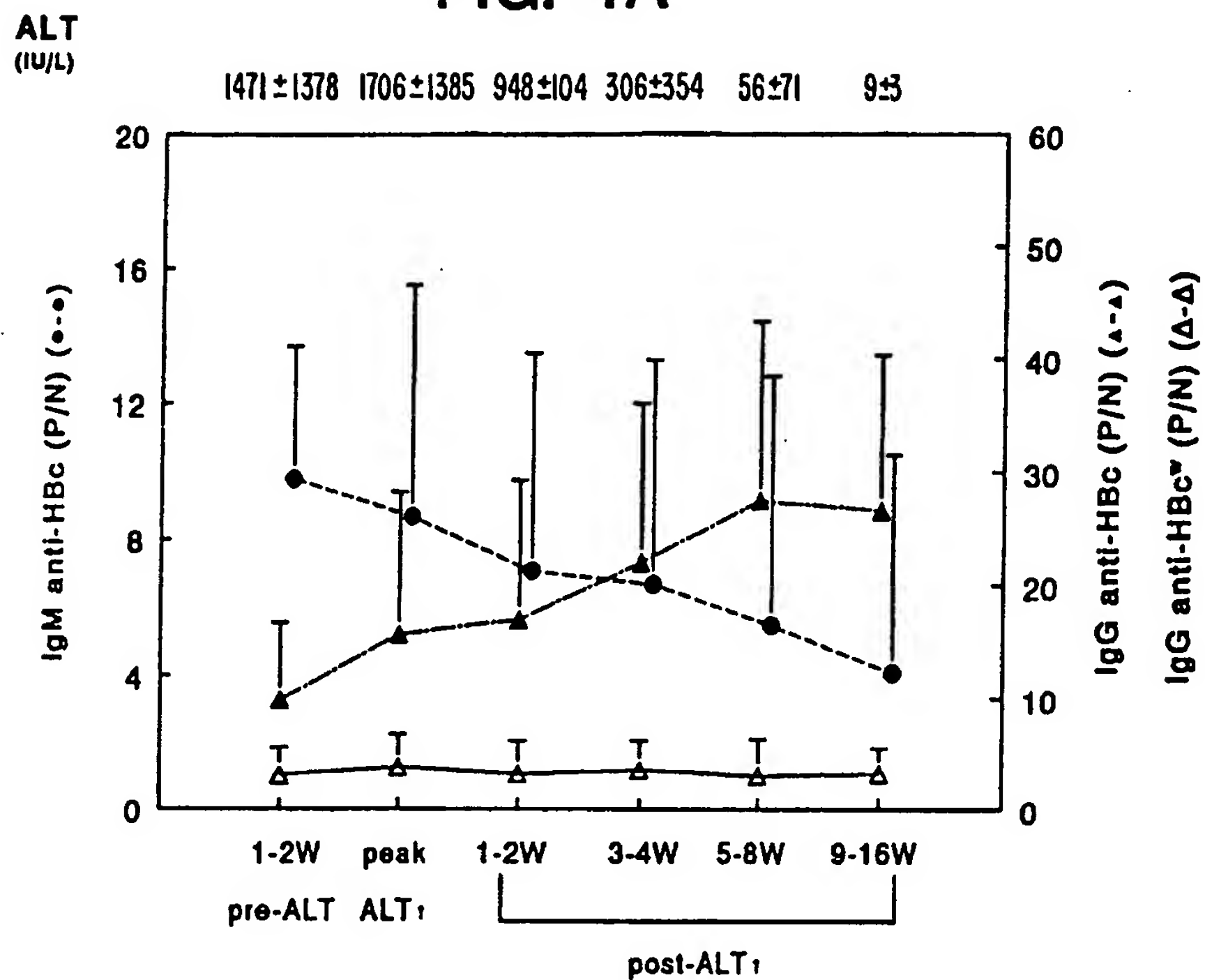
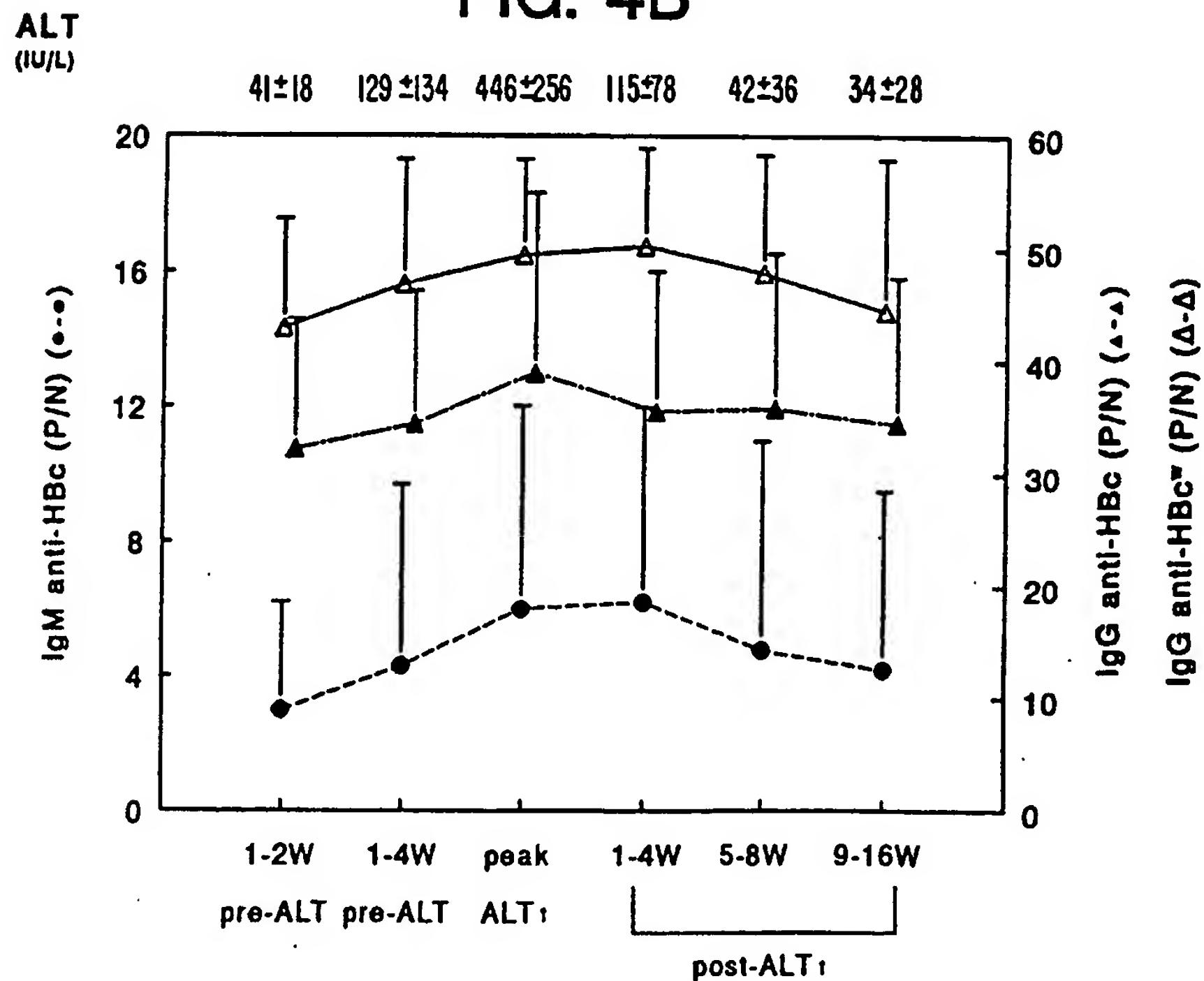


FIG. 4B



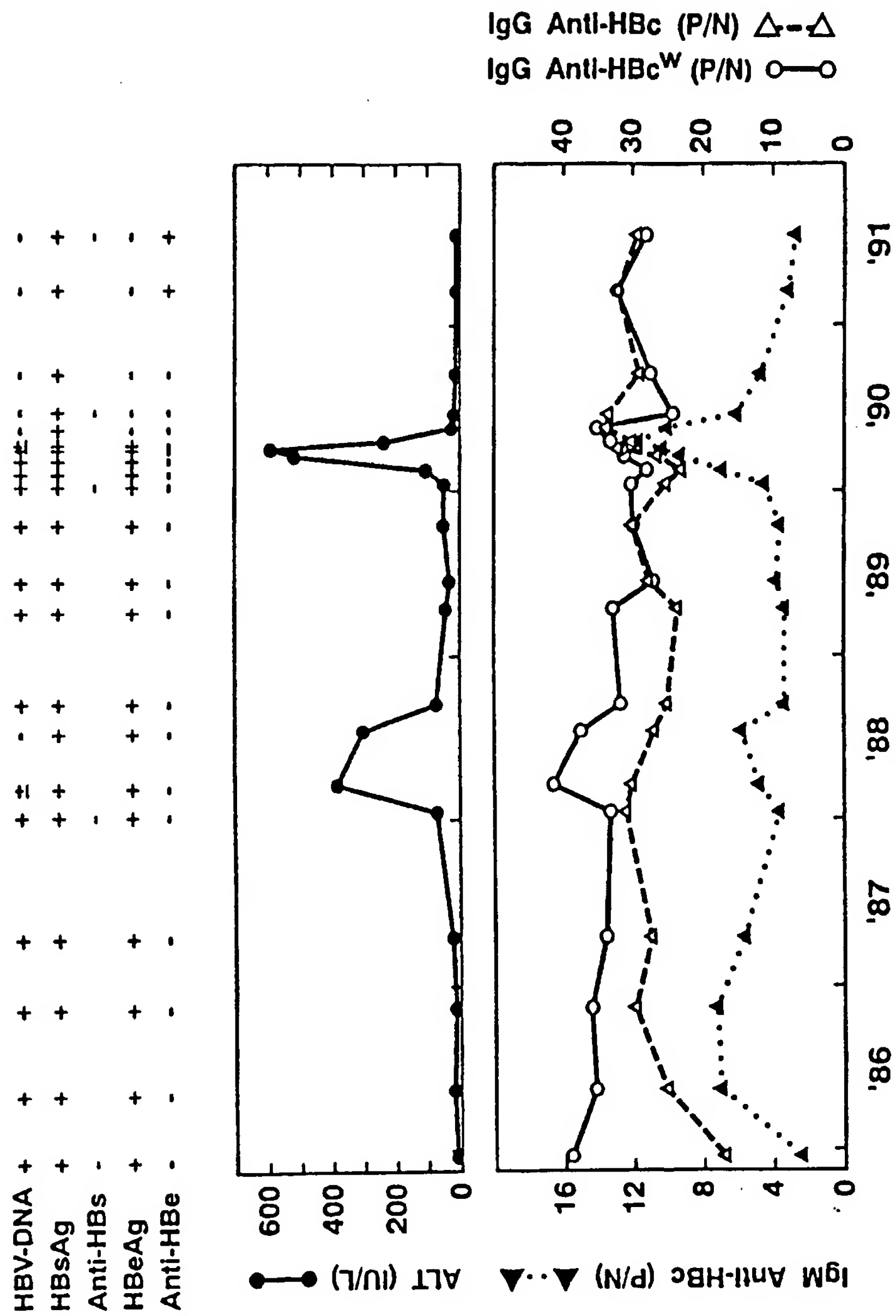


FIG. 5

FIG. 6A

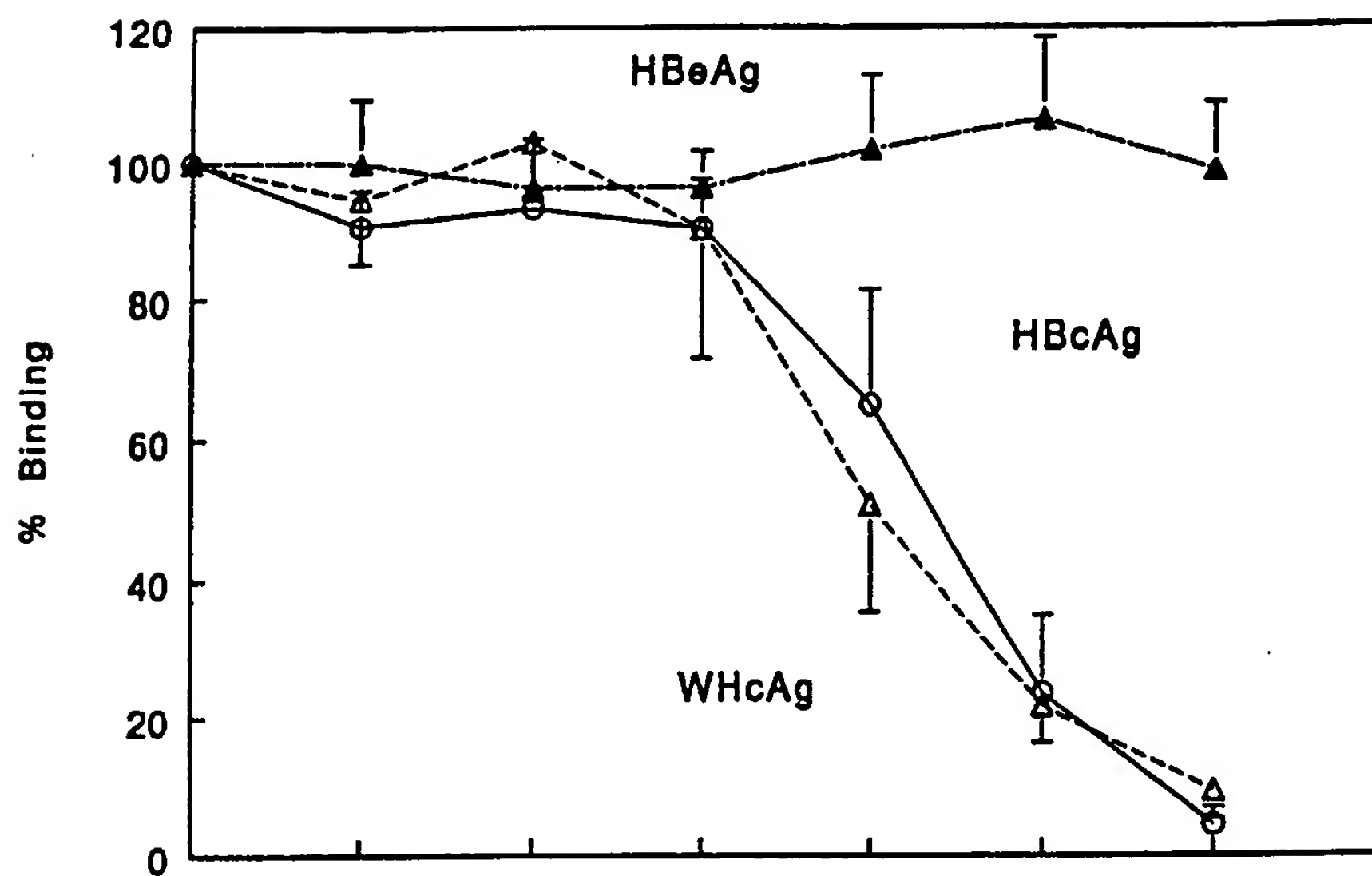
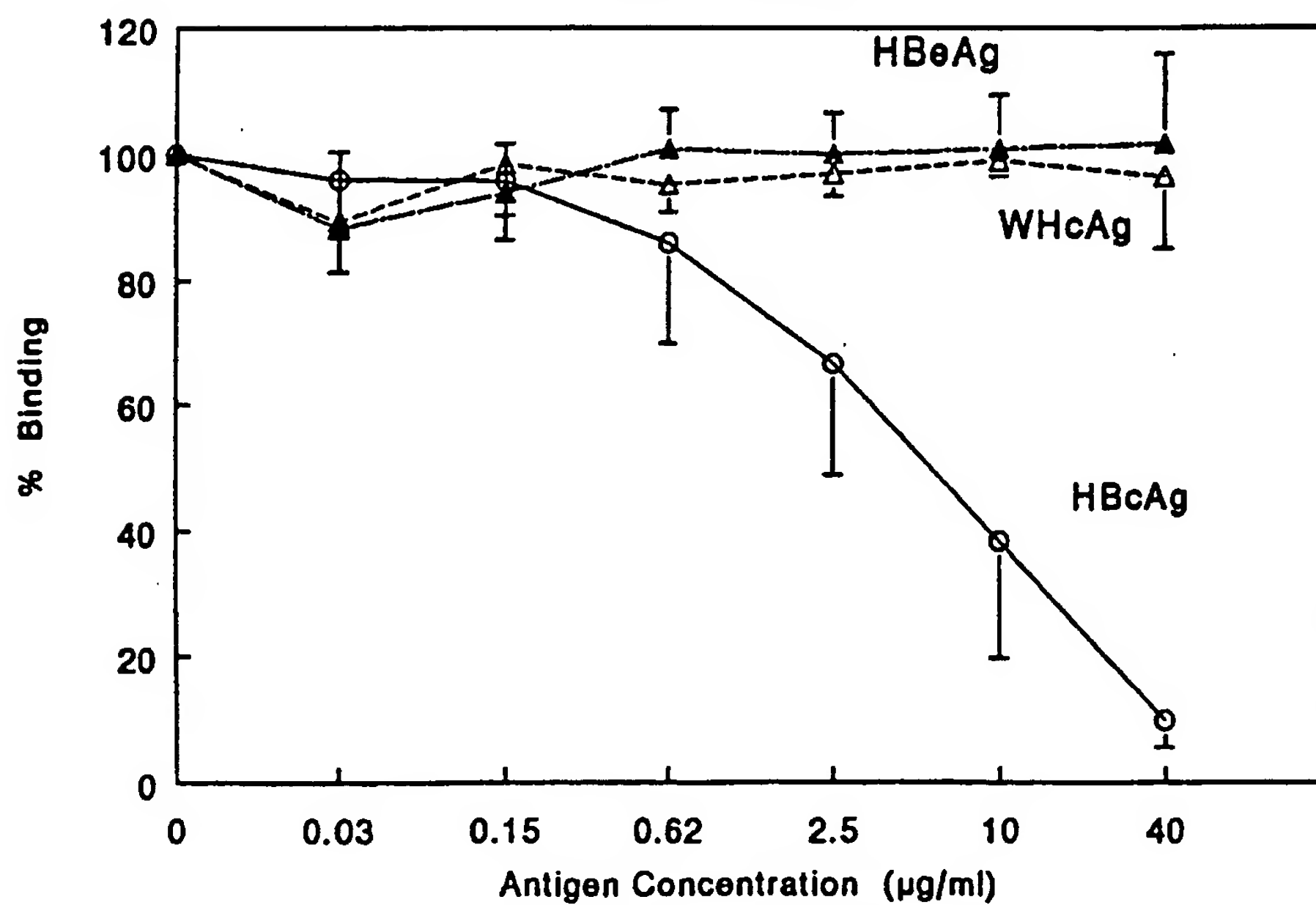
Anti-HBc^w

FIG. 6B

Anti-HBc



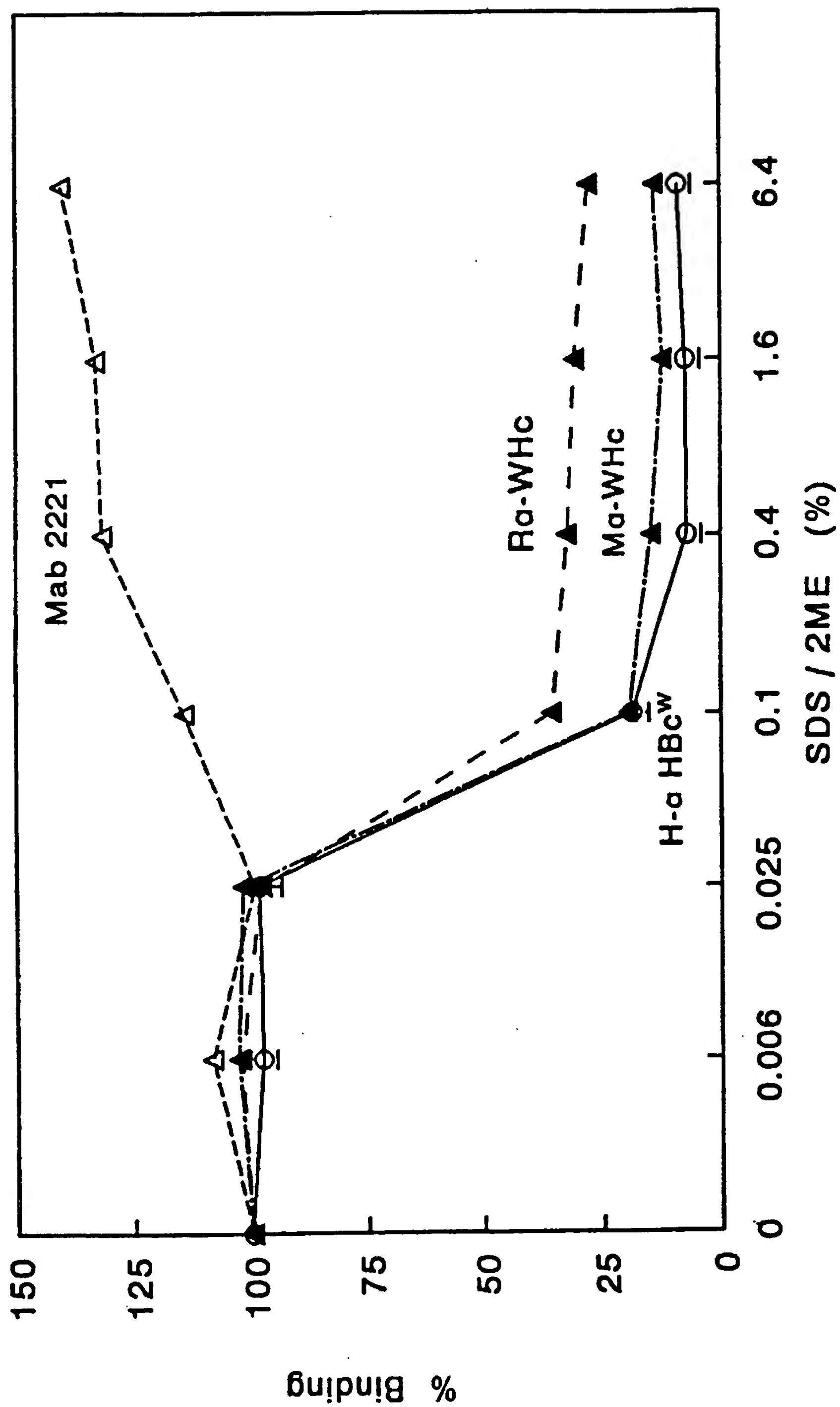


FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/04061

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/70

US CL : 435/5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5; 436/518, 820

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| A | Journal of Virology, Volume 52, No. 1, issued October 1984, A. Ponzetto et al., "Core Antigen and Antibody in Woodchucks After Infection with Woodchuck Hepatitis Virus," pages 70-76, see entire document. | 1-11, 20-22 |
| A, P | Hepatology, Vol. 20, No. 2, issued August 1994, T. Michalak et al., "Molecular Species of Hepadnavirus Core and Envelope Polypeptides in Hepatocyte Plasma Membrane of Woodchucks with Acute and Chronic Viral Hepatitis," pages 275-286, see entire document. | 1-11, 20-22 |



Further documents are listed in the continuation of Box C.



See patent family annex.

| | | | |
|-----|---|-----|--|
| * . | Special categories of cited documents: | *I* | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| *A* | document defining the general state of the art which is not considered to be of particular relevance | *X* | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| *E* | earlier document published on or after the international filing date | *Y* | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| *L* | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | *Z* | document member of the same patent family |
| *O* | document referring to an oral disclosure, use, exhibition or other means | | |
| *P* | document published prior to the international filing date but later than the priority date claimed | | |

Date of the actual completion of the international search

19 JUNE 1995

Date of mailing of the international search report

12 JUL 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

DONNA C. WORTMAN, Ph.D.

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/04061

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| Y | Journal of Clinical Investigation, Vol. 91, issued June 1993, T. Maruyama et al., "The Serology of Chronic Hepatitis B Infection Revisited," pages 2586-2595, see entire document. | 12-19, 23-26 |
| Y | Journal of Immunological Methods, Vol. 155, issued 1992, T. Maruyama et al., "Use of anti-peptide antibodies for the design of antigen-specific immune complex assays," pages 65-75, see entire document. | 12-19, 23-26 |
| A | Journal of General Virology, Vol. 64, issued 1983, L. M. Stannard et al., "Antigenic Cross-reactions between Woodchuck Hepatitis Virus and Human Hepatitis B Virus Shown by Immune Electron Microscopy," pages 975-980, especially page 978. | 1-11 |
| --- | | ----- |
| X | | 20-22 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/04061

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/04061

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-11 and 20-22, drawn to method of diagnosing chronic viral infection using woodchuck hepatitis B core antigen.

Group II, claims 12-19 and 23-26, drawn to method of diagnosing chronic viral infection using a hepatitis B core antigen, antibody to HBeAg/anti-HBe immune complex, and antibody to HBsAg/anti-HBs immune complex.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The two distinct methods of diagnosis do not share a special technical feature. The first method requires woodchuck hepatitis B core antigen as a reagent, while the second method requires a combination of antigens not including woodchuck hepatitis B core antigen. The two methods do not have the same or corresponding special technical feature because the second method does not require use of woodchuck hepatitis B core antigen. The claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.